LAMININ-5 DEGRADATION DUE TO MUSTARD IN CULTURED NORMAL HUMAN EPIDERMAL KERATINOCYTES (NHEK)

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ABSTRACT

Laminin-5, a heterotrimer of laminin α3, β3, and γ2 chains, is essential for a stable attachment of the epidermis to the dermis. Laminin-5 facilitates the recovery of damaged skin. We studied the effects of both mustards on laminin-5 by Western blotting analysis. We observed that in NHEK, mustards degrade laminin-5. Calmodulin antagonist, W7 or the serine protease inhibitor, TLCK prior to mustard exposure prevented mustard-induced degradation of laminin-5. The results provide important information on the mechanism of laminin-5 degradation by mustards and thus will be useful for developing strategies to prevent skin damage due to mustard exposure.

INTRODUCTION

Di (2-chloroethyl) sulfide (HD) has been used as a chemical weapon, the skin being a principal target. HD is a threat to both civilians and military personnel. HD and HN_2 (N-methyl-2, 2'-dichlorodiethylamine) are known to behave in similar manners. Both HD and HN_2 are potent and relatively nonspecific bifunctional alkylating agents, which, in aqueous solutions, are capable of reacting with a host of compounds that are vital to the living cells (1). The basement membrane between the epidermis and the dermis contains unique structures that maintain the attachment of the epidermis. The components of the attachment complex provide links to the intracellular intermediate filament network of basal keratinocytes and to the extracellular matrix of the papillary dermis. One of the key components of anchoring is laminin-5 (2). Laminin-5, a heterotrimer of laminin-5 α3, β3 and γ2 chains, is a major keratinocyte adhesion ligand, and is found in the lamina lucida subregion of the epidermal basement membrane of the skin. Laminin-5 colocalizes with the anchoring filament. Laminin-5 promotes keratinocyte adhesion, migration, and scatting of various types of cells (3-4). Previous studies have shown that laminin-5 is essential for epidermal attachment, because mutations in the genes encoding the laminin-5 chains underlie the severe blistering phenotype of Herlitz' junctional epidermolysis bullosa (5). In this study, we investigated how laminin-5 may be involved in mustard induced blister formation by using low (multiple 1 μM and 5 μM) and/or chronic (24 hours, 48 hours) doses compared with a single dose (300 μM, 16 hours).

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METHODS

Normal human epidermal keratinocyte (NHEK) were used in this investigation. Cells were exposed to HD at USAMRICD according to their approved protocol at low dose (multiple 1 μM and 5 μM; chronic 24 and 48 hours) conditions and the results were compared with a single high dose (300 μM, 16 hours). Cell lysates enriched in laminin-5 were collected following the instructions (6). Briefly, NHEK were detached by scraping in lysis buffer containing 10 mM Tris.HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 5mM EDTA, 1mM phenylmethylsulfonyl fluoride, 0.28 unit/ml aprotinin, 50 ug/ml leupeptin. Proteins were separated by 4-12% SDS-polyacrymide gel electrophoresis and then transferred to a polyvinylidence difluoride (PVDF) membrane. Blots were incubated with antibodies against laminin-5 β3, laminin-5 γ2 (Santa Cruz Biotechnology) and then probed with a horseradish peroxidase-conjugated second antibody. Detection was performed by using enhanced chemiluminescence (ECL kit, Amersham Pharmacia). Also laminin-5 distribution and content were determined by an immunofluorescence method (7). Briefly, cells grown on glass coverslips were fixed in 3.7% para-formaldehyde, washed with phosphate buffered saline (PBS), and blocked with normal goat serum (5%) for 1 hour at room temperature (RT), then incubated with primary antibody for 1 hour. After 3 times for 5 minutes each time washes, cells were reacted with the secondary rhodamine (TRITC)-conjugated secondary antibody for 1 hr RT and washed as before. Coverslips were mounted in mounting solution (SIGMA). Cells were viewed with a Bio-Rad laser confocal system attached to an Olympus microscope.

RESULTS

First, we studied whether laminin-5, laminin-5 β3, and laminin-5 γ2 exist in normal human epidermal keratinocyte. We use Western blot analysis of laminin-5 and laminin-5 β3, laminin-5 γ2 in NHEK. Measurement of laminin-5, laminin-5 β3, and laminin-5 γ2 in NHEK shows that laminin-5 exists in two molecular weights 440 kDa, 400 kDa; laminin-5 β3 exists in two molecular weights 140 kDa, 100 kDa and Laminin-5 γ2 exists in two molecular weights 155 kDa, 105 kDa (*Fig. 1*). Immunofluorescence (Rhodamine-conjugated secondary antibody) shows that laminin-5 distribution is in cell membrane and matrix *(Fig. 1)*. Then we studied whether laminin-5, laminin-5 β3, and laminin-5 degraded after both HD and HN_2 treatment. HN_2 (100 μM, 300 μM) induces laminin-5 γ2 (155 kDa) degradation at 16 hrs after treatment. Addition of 25 μM N-(6-Aminohexyl)-5-chloro-1-Naphalene-Sulfonamide (W7) and 10 μM Na-p-Tosyl-L-Lysine Chloro-methyl ketone (TLCK) 1 hr before HN_2 treatment, blocks laminin-5 γ2 degradation partially *(Fig. 2)*.

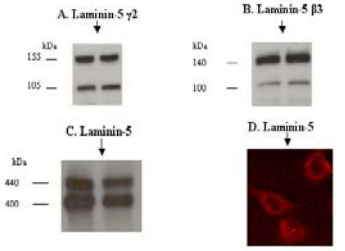

Figure 1. Western blot & Immunofluorescence (Rhodamine-conjugated secondary antibody) analysis of laminin-5 and laminin-5 β3, γ2 in NHEK.

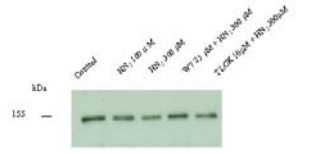

Figure 2. Effect of inhibitors N-(6-Aminohexyl)-5-chloro-1-Naphalene-Sulfonamide (W7) and Na-p-Tosyl-L-Lysine Chloro-methyl ketone (TLCK) on laminin-5 degradation after HN_2 treatment.

HD (300 μM, 16 hours) causes laminin-5 β3 (140kDa, 100 kDa) & γ2 (155 kDa, 105 kDa) degradation. Addition of 25 μM N-(6-Aminohexyl)-5-chloro-1-Naphalene-Sulfonamide (W7) 1 hr before HD treatment, blocks laminin-5 β3 & γ2 degradation partially *(Fig. 3)*. We also use Immunofluorescence analysis of laminin-5 and laminin-5 γ2 degradation. It shows that Nitrogen mustard treatment causes laminin-5 degradationat at 16 hours after treatment *(Fig. 4)*.

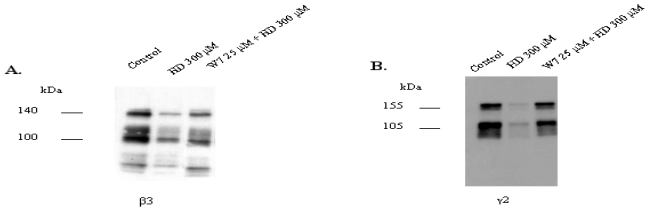

Figure 3. Effect of inhibitor N-(6-Aminohexyl)-5-chloro-1-Naphalene-Sulfonamide (W7) on laminin-5 degradation after HD treatment.

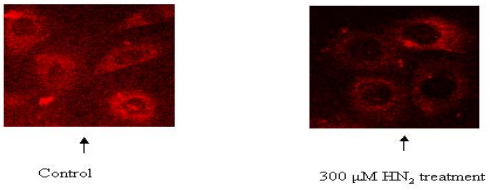

Figure 4. Immunofluorescence analysis of laminin-5 and laminin-5 γ2 degradation.

Next we investigated whether laminin-5 were degraded by HD at low dose and chronic time treatment condition. At low dose (1μM and 5 μM, 24 hrs) HD treatment, both laminin-5 γ2 (155 kDa) & β3 (140 kDa) are degraded at concentration 1μM×3, 5μM×1 and 5μM×3 *(Fig. 5)*. At low dose (1μM and 5 μM, 48 hrs) HD treatment, both laminin-5 γ2 (155 kDa) & β3 (140 kDa) are degraded at concentration 5μM×1 and 5μM×6 *(Fig. 6)*.

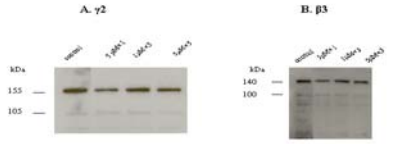

Figure 5. Low dose (1μM and 5 μM) and chronic (24 hours) exposure of HD on laminin-5 γ2 and laminin-5 β3 dergadation.

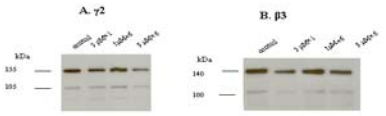

Figure 6. Low dose (1μM and 5 μM) and chronic (48 hours) exposure of HD on laminin-5 γ2 and laminin-5 β3 dergadation.

Actin was not degraded at these concentrations *(Fig. 7)*. At low-dose HD (5 μM, 24 hrs and 48 hours) both laminin-5 (440 kDa) & laminin-5 γ2 (155 kDa) were degraded under non-denaturing gel *(Fig. 8)*.

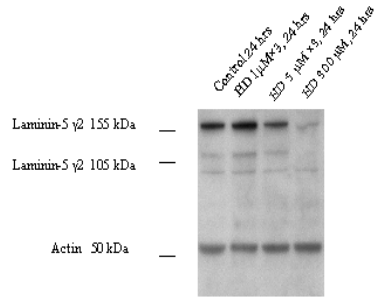

Figure 7. Laminin-5 γ2 (155 kDa) was degraded at HD concentrations 5μM×3 and 300 μM. Actin was not degraded at these concentrations.

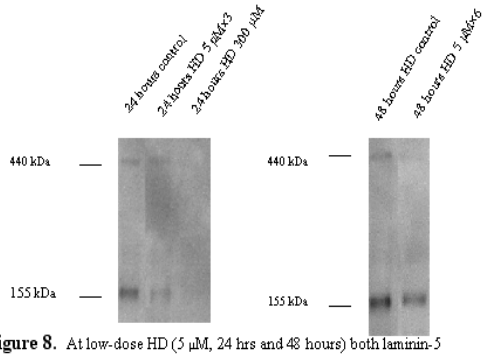

Figure 8. At low-dose HD (5 μM, 24 hrs and 48 hours) both laminin-5 (440 kDa) & laminin-5 γ2 (155 kDa) were degraded under non-denaturing gel.

CONCLUSIONS

Culture of normal human epidermal keratinocytes in KGM complete medium plus cell growth factors produced sufficient materials for the laminin-5 studies. Laminin-5 (440 kDa, 400 kDa), laminin-5 γ2 (155 kDa, 105 kDa), and laminin-5 β3 (140 kDa, 100 kDa), extracted from NHEK were reliably detected by Western blot method. Laminin-5, laminin-5 γ2, β3 chains are some of the proteins degraded due to HN_2 and HD treatment in NHEK at both low chronic dose and single high dose (300 μM). Exposure of NHEK to HN_2 decreased laminin-5 as seen by the immunofluorescence method. The

observations that the calmodulin antagonist W7 and trypsin-like serine protease inhibitor TLCK blocked laminin-5 degradation in mustard treated NHEK suggest that mustards stimulate a calcium/calmodulin-dependent serine protease that degrades laminin-5. Laminin-5 plays a key role in cellular migration and plays an important role in epidermal-dermal adhesion. In addition, the presence of laminin-5 appears to play a role in the formation of hemidesmosomes. Thus, mustard-induced degradation of laminin-5 potentially may inhibit both cellular migration and adhesion, and this might contribute to vesication.

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